

Human Somatic Mutation Assays As Biomarkers of Carcinogenesis

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This paper describes four assays that detect somatic gene mutations in humans: the hypoxanthine-guanine phosphoribosyl transferase assay, the glycophorin A assay, the HLA-A assay, and the sickle cell hemoglobin assay. Somatic gene mutation can be considered a biomarker of carcinogenesis, and assays for somatic mutation may assist epidemiologists in studies that attempt to identify factors associated with increased risks of cancer. Practical aspects of the use of these assays are discussed.

Introduction

Cancer development is a multistep process that may take years in humans. Critical steps in the process appear to be the production of stable changes in the genetic material, called mutations, and subsequent cell proliferation that produces daughter cells with mutated DNA.

A number of mutational events are thought to be necessary to convert a normal cell into a malignant cancer cell. For example, sequential mutational changes have been identified in tissues of the colon that are related to clinical stages in the progression of colon cancer (1). The development of somatic mutation assays as biomarkers of the carcinogenic process may, therefore, help epidemiologists link carcinogenic exposures to cancer outcome in humans by looking at events early on in the cancer process. This paper provides an overview of current human somatic gene mutation assays and discusses some of the practical aspects involved in their application to epidemiological study populations.

Relationship between Mutation and Cancer

The lines of evidence that link somatic mutation with cancer can be broadly summarized as follows.

a) Cancers often arise in proliferating tissues in contact with the environment, e.g., the mouth, gut, skin, and lungs (2). In a tissue in which there is rapid cell proliferation, damage to DNA may not be completely repaired before cell division occurs. The damage may then be passed on and made permanent in DNA strands of the daughter cells.

b) Many carcinogens bind to or cause mutation in DNA. For example, benzo[a]pyrene DNA adducts have been detected in cells exposed to the carcinogen benzo[a]pyrene (3).

c) DNA from tumor cells can "transform" normal cells. When DNA from tumor cells was transferred to normal cells, the normal cells became transformed, i.e., exhibited characteristics of tumor cells (4).

d) Stable, nonrandom changes in the genetic material are associated with certain cancers. For example, most cases of chronic myelogenous leukemia exhibit a characteristic "Philadelphia" chromosome, which is the product of a reciprocal translocation between chromosomes 22 and 9 (5).

e) A tumor probably arises from the clonal expansion of a single cell, based upon observations of monoclonal X-inactivation patterns in tumors (6).

f) Certain genetic conditions with a high risk of cancer have been shown to have defects in DNA repair processes, e.g., ataxia telangiectasia (7) and Bloom syndrome (8).

Mutation can have consequences other than cancer. Mutation can cause birth defects or mutations transmitted in the germ line (9), and it has been implicated in the causation of disease, e.g., atherosclerosis (10). Also, aging may be due to the accumulation of mutations with the years (11). Biomarkers of mutation may, therefore, be useful as indicators of effects other than cancer. It should be remembered, however, that mutation also may have no effect, since much of DNA has no known function (12).

Human Mutation Assays

Assays that measure genetic damage in humans can be divided into two broad categories: assays of change at the level of gross chromosomal structure and assays of change at the level of the gene. The DNA in the nucleus is packaged into chromosomes, which are linear DNA molecules with secondary and tertiary structure. Genes are short pieces of DNA that code for RNA and proteins, and a gene is very small compared to a chromosome: genes make up approximately 3% of a chromosome, and many genes reside on each chromosome (13).

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Assays of change in chromosome structure include the chromosome aberration assay, which scores the number of abnormal, broken, or missing chromosomes in metaphase cells (14); the SCE assay, which counts sister chromatid exchanges (exchanges of identical pieces of chromosomes in duplicated sister chromatids during cell replication) in metaphase cells (15); and the micronucleus assay, which measures the frequency of micronucleus formation (chromosomes or fragments of chromosomes lost to the cytoplasm during cell division) (16). These assays are usually performed in white blood cells (lymphocytes). Such assays may reflect damage as well as mutation, since not all of the events scored reflect a heritable change that is transmitted from a cell to a daughter cell, the definition of mutation. This article focuses on human somatic mutation assays at the level of the gene.

There are practical constraints to measuring gene mutations in humans. First, the approach must use an accessible tissue. Second, the method must be able to identify mutant cells and, since mutation is a rare event, to find and enumerate these cells against a high background number of normal cells.

Only a small number of human mutation assays have been developed to date because assays on people must use normal human genetic material. This is in contrast to the large number of assays that have been developed in nonhuman species, where the application of techniques of genetic engineering or selection can be used to make detection of mutants relatively simple. All of the current human mutation assays use blood cells, and all use a change in or absence of a normally functioning protein to detect mutations in the gene coding for the protein.

In using these assays as biomarkers of the carcinogenic process, it is preferable to measure changes in genes known to be important in cancer. Many genes have been identified to date, both oncogenes (onco- for tumor or mass) which have been shown to be activated by specific mutation in certain cancers, and antioncogenes or tumor-suppressor genes, which have been shown to be deactivated by mutation (17). Even in the cases where it is possible to detect a change in these "cancer" genes, however, there is currently no method for identifying the mutant cells against the background of normal cells. Identifying cells with mutations in cancer genes using present technology would require screening thousands or millions of colonies grown from individual cells, and this is not practical. Current human gene mutation assays instead screen for mutations that can be easily identified and selected for by a change or absence of a normal protein produced by specific genes. The mutation frequency (and type) produced in these genes is then used as a surrogate for the amount and types of mutations potentially found in cancer genes.

The HPRT Assay

The hypoxanthine-guanine phosphoribosyl transferase (HRPT) assay was the first human somatic gene mutation assay to be developed (18–20). This assay identifies and selects (finds against the background of normal cells) mutant cells in one step by taking advantage of the biochemical pathways by which a cell synthesizes DNA.

In cells, DNA is synthesized in two ways, either from nucleotide bases (adenine, thymine, guanine, and cytosine, which make up the genetic code) made *de novo*, or from bases recycled from degraded DNA by the so-called "salvage" pathways. HPRT is

one of the enzymes that recycles nucleotide bases. White blood cells that have mutations at the *hprt* gene that lead to a nonfunctioning HPRT protein can be detected by adding a toxic analog (6-thioguanine [6TG]) of the nucleotide bases to the cells. Normal white blood cells incorporate the toxic analog into newly synthesized DNA, leading to cell death (Fig. 1a). Mutant cells that have a nonfunctioning HPRT enzyme do not incorporate the toxic analog and survive (Fig. 1b). For the HPRT mutation assay, white blood cells isolated from a human blood sample are cultured *in vitro* with the 6TG, and the number of surviving mutant cells is determined after a period of cell growth (1–3 weeks) by counting the number of cell colonies. Counting is accomplished either by an autoradiographic method or a cell cloning technique.

Mutants can be detected in this assay because only one functional copy of the *hprt* gene is present per cell. If two copies of the gene were present, the loss of one copy would be hard to detect, as the other copy would probably supply the missing function. Only one copy of the *hprt* gene is present because it is located on the X chromosome, and there is only one active X chromosome per cell. Humans have 22 pairs of autosomal (i.e., nonsex) chromosomes and one pair of sex chromosomes: females have two X chromosomes and males have one X and one Y chromosome. Females randomly inactivate one of the two X chromosomes in each cell during development so that females will have the same amount of gene product from genes residing on the X chromosome as do males.

Because only one chromosome carries the functional *hprt* gene, the HPRT assay probably does not detect mutations requiring interaction between both chromosomes of a pair. Recent evidence from cancer biology indicates that chromosome-chromosome interactions could be an important subclass of mutations in cancer (17).

Chromosomal-Chromosomal Mutational Mechanisms in Cancer

Studies of genetic changes found in several cancers suggest that chromosome-chromosome mutational mechanisms are of importance in cancer. The first clear evidence came from studies of the disease retinoblastoma, a childhood cancer of the eye. A prerequisite for formation of the tumor appears to be that both copies of a gene (called *rb* for retinoblastoma, one copy on each chromosome 13) have been altered and have lost the ability to produce a protein that suppresses cancer. The *rb* gene is a member of the class of recently discovered tumor-suppressor genes. The children with the hereditary (from a parent or a *de novo* germ line mutation) form of retinoblastoma have one constitutive defective copy of this gene, and tumors apparently arise from the loss of the remaining functional copy of *rb* during development (21,22).

In order to determine the molecular mechanisms by which the functional copy of *rb* was lost, the tumor DNA was analyzed by new molecular techniques. It was found that two general classes of mutational events led to the loss of the functional *rb* gene, called here gene-loss/inactivating and gene-duplicating mutations (Fig. 2) "Gene-loss/inactivating" mutations refer to changes to the functional *rb* gene that eliminate gene function, such as deletion of part or all of the gene, or point mutations that change the

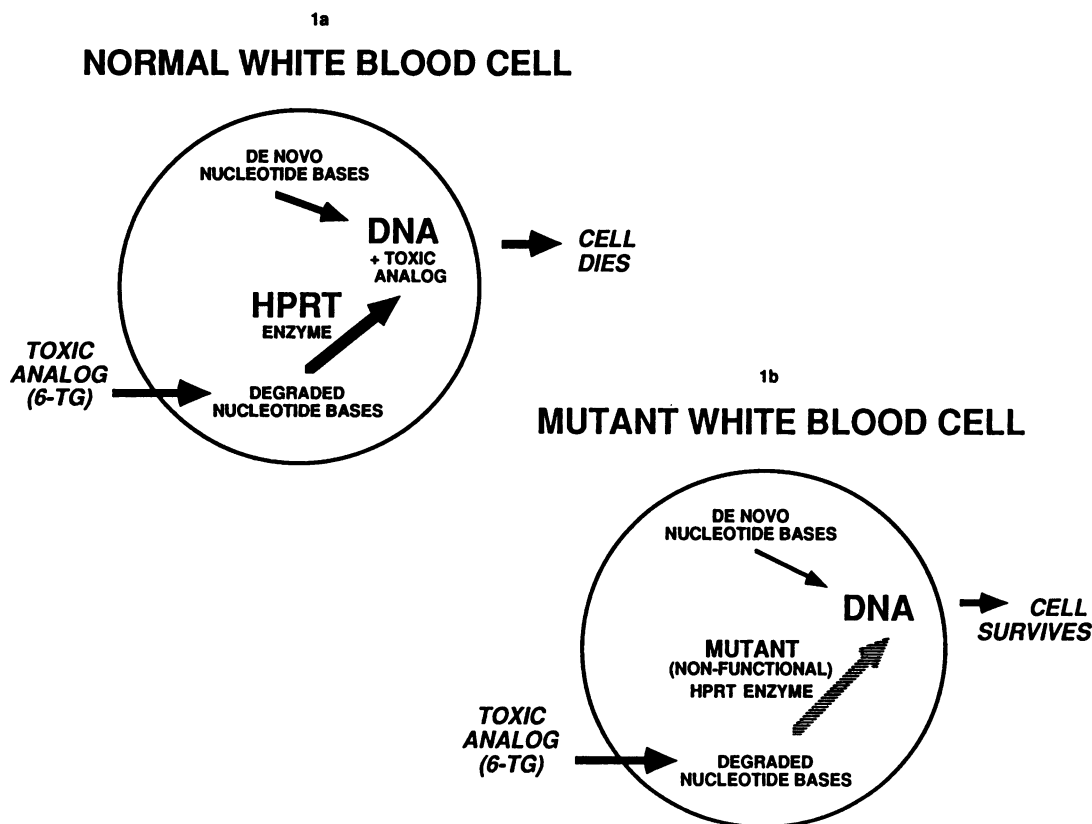


FIGURE 1. The HPRT mutation assay selects mutants from T-lymphocytes by exploiting a biochemical pathway of DNA synthesis. DNA is made from new or recycled nucleotide bases. HPRT is one of the enzymes in the salvage, or recycling, pathway. In nonmutant cells with functional HPRT enzyme, a toxic analog of nucleotide bases (6-thioguanine) is incorporated into DNA, leading to cell death. In mutant cells that lack a functional HPRT enzyme, the toxic analog is not incorporated, and the cells survive.

base sequence of DNA. Mutations of this type are detected by the HPRT assay since they require that only one copy of the gene be present. "Gene-duplicating" mutations are those in which the inherited dysfunctional copy of the gene on the other chromosome "replaces" the good copy of the gene. Mitotic recombination or reduplication of the chromosome carrying the dysfunctional gene are gene-duplicating mechanisms, and they have been demonstrated in many of the *rb* cases (23). Such gene-duplicating events obviously require that both chromosomes of a pair be present.

Two recently developed human mutation assays, the glycoporphin A assay (GPA) and the HLA-A assays, are able to detect chromosome-chromosome interactions because the genes studied are located on autosomal (nonsex) chromosomes. (This means there are two copies of each gene, one on each of the paired chromosomes.) Both methods assay for the loss of a surface protein on cells heterozygous for the gene making the protein. The cells must be heterozygous because a mutation at one allele (form of the gene) in a heterozygous cell will lead to complete loss of that form of the normal protein product from the cell. In a homozygous individual, a mutation in one allele would probably not affect the protein produced by the other allele. Since mutant cells are detected by the loss of the protein, these cells would not be detected as mutants.

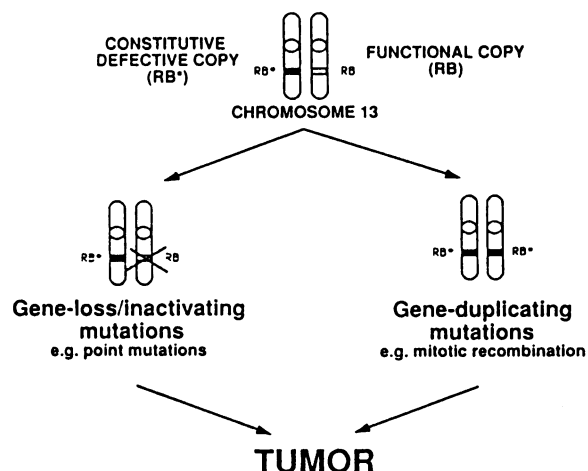


FIGURE 2. Mutational mechanisms generating loss of tumor-suppressor genes in retinoblastoma. Retinoblastoma apparently arises due to loss of both functional copies of the *rb* gene on chromosome 13. Analysis of tumor DNA indicates that two general types of mutational mechanisms, gene loss/inactivating and gene duplicating, lead to loss of the remaining functional *rb* gene in children with a constitutive defective *rb* gene.

GPA Assay

The GPA assay measures cells that have lost one form of the GPA protein present on the surface of red blood cells (23,24). The gene has two alleles called M and N and is located on chromosome 4, so a heterozygous individual will have one chromosome 4 with an M allele of the GPA gene and one with an N allele of the GPA gene (Fig. 3). Normal red blood cells from this individual will have both M and N glycoproteins on their cell surface. About 50% of the population is MN heterozygous, i.e., of MN blood type.

For the GPA mutation assay, red blood cells from a person with blood type MN are reacted with anti-glycoprotein antibodies that can discriminate between the M and N forms of glycoprotein. The blood cells are first fixed (surface proteins are cross-linked) to stabilize the cells and prevent agglutination when reacted with antibodies. The antibodies are labeled with fluorescent molecules—anti-N with green and anti-M with red. Thus, a normal red blood cell from a person heterozygous for the MN allele will fluoresce red and green. Blood cells that have suffered a mutation in the GPA M gene that prevents proper expression of GPA M on the cell surface will fail to bind antibody to M and will fluoresce green only (Fig. 3). The antibody-bound cells are analyzed by a flow cytometer, which measures fluorescence from all cells and counts the number of green-only mutant cells.

Two classes of variant red blood cells can be detected, $N\phi$ and

NN. $N\phi$ cells have the normal amount of N-GPA on the cell surface and no detectable anti-M antibody binding and presumably arise from gene-loss/inactivating mutations. NN cells have twice the normal amount of N on the cell surface and no M-binding, and presumably arise from gene-duplicating mutations (Fig. 3). Finding mutant cells by screening all cells (as opposed to the HPRT assay where all normal cells are eliminated) is made possible because flow cytometers can analyze cells very rapidly. In the GPA assay, fluorescence from each of five million cells can be measured in 20 min and the mutant cells enumerated.

The GPA assay has the advantage that it can detect chromosome-chromosome interactions such as mitotic recombination. It is limited in that the red blood cell lacks a nucleus, and this precludes any investigation of mutational mechanisms at the molecular level. In addition, it cannot be proven that variant cells are mutant cells, although evidence suggests that this is the case (24).

HLA-A Assay

The recently developed HLA-A assay (25) is performed on white blood cells, which contain DNA, and therefore the assay can be used to provide detailed information on mutational mechanism. HLA-A is a surface protein found on most nucleated cells in the body and is involved in the immune response and self versus nonself discrimination (26). The HLA-A gene is on

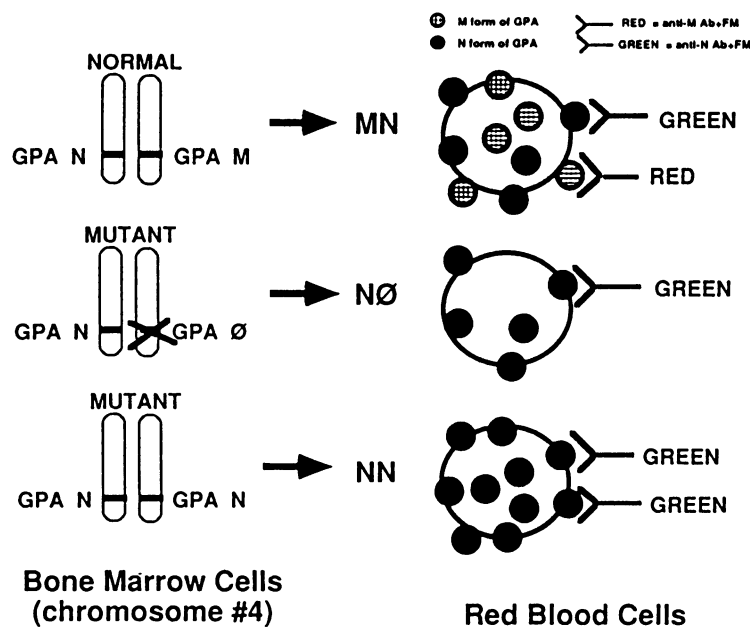


FIGURE 3. The GPA assay requires blood samples from persons heterozygous for glycoprotein A, a cell surface protein on red blood cells. In a normal red blood precursor cell from a GPA heterozygote, one chromosome of the chromosome 4 pair carries the M form of GPA and one the N form (left). Mutant cells may arise from gene-loss/inactivating mutations (called $N\phi$) and gene-duplicating mutations (called NN) (left). The right portion of the figure shows a normal heterozygous red blood cell with both M and N blood group proteins on the cell surface, and mutant cells with normal ($N\phi$) or twice normal (NN) amounts of N GPA, but which lack normal M GPA. Normal and mutant cells are reacted with anti-GPA M and anti-GPA N antibodies labeled with fluorescent molecules, anti-M with red and anti-N with green. Fluorescence of each cell is measured by flow cytometry, and the number of green-only mutants, both $N\phi$ and NN, is determined for each sample.

chromosome 6 and is one of the most polymorphic loci known, which means that most people are heterozygous for the HLA-A protein, i.e., have more than one form on their cells.

The HLA-A human somatic mutation assay is similar to the GPA assay in that mutants are detected by lack of antibody binding to a cell surface protein. In order to detect mutants, however, normal cells are eliminated as in the HPRT assay, rather than counted as in the GPA assay.

To determine HLA-A mutant frequencies for an individual, white blood cells are isolated from a blood sample and typed with HLA-A antibodies. The assay currently works on individuals heterozygous for A2 or A3 forms (alleles) of HLA-A. More than 60% of an average population is heterozygous for one or the other of these alleles. Blood from an A2Ax or A3Ax blood type individual (x = other allele) is incubated with the A2 or A3 antibody, and the cells are treated with complement. Complement is a complex of serum proteins that is part of the body's natural immune defenses and acts to kill antibody-covered cells. The addition of complement, therefore, kills the normal A2 or A3 antibody-covered cells, and mutant cells with altered or missing HLA-A protein fail to bind the antibody and survive (Fig. 4). The cells are plated, and the surviving cells are grown into clones, which are counted to obtain a mutant frequency after correcting for growth on nonselected plates. The clones can then be analyzed to characterize the nature of the mutation in the cell. In a sample of normal individuals, mitotic recombination was responsible for an average of one-third of the mutations observed (27).

Hb-S and Other Assays

A fourth human somatic mutation assay, Hb-S, detects the production of a mutant form of hemoglobin (Hb-S) caused by a specific point mutation in one of the Hb genes (28–30). Hb-S is the hemoglobin responsible for sickle cell anemia. Fluorescently labeled antibodies for the mutated Hb are added to blood preparations fixed on a slide. The slide is then scanned by microscopy (automated image analysis) to detect anti-Hb-S antibody binding (28). This assay detects only one specific mutation, a change of the base adenine to thymine. The assay therefore does not detect a wide range of mutational mechanisms and so has a much lower mutant frequency than is observed in either the HPRT, GPA, or HLA-A assays. However, modifications are being introduced that will increase the number of mutations detected (28).

Other assays that are currently under development look at changes in the DNA directly rather than using a cellular protein selection process (31). Such assays hold promise for the future because they could be performed on many more tissue types, as they do not require cell growth and do not require the gene product to be expressed. This could allow the detection of mutations in tissues thought to be directly targeted by a certain exposure. Current somatic mutation assays are all performed on one tissue, the blood, and there are uncertainties inherent in extrapolating from mutations in a nontarget tissue to mutations in a target tissue. DNA assays also have the potential to be used on stored samples. In addition, the bias inherent in the phenotype selection approach (which detects only mutations that produce an altered protein) would be eliminated, since these assays would be able to detect mutations even if they did not lead to an altered protein.

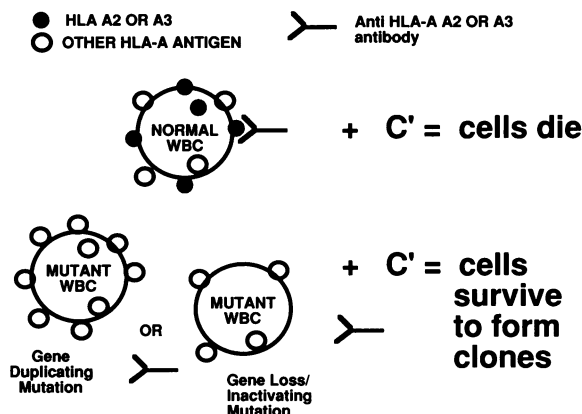


FIGURE 4. The HLA-A assay requires blood samples from subjects heterozygous at the HLA-A gene, a gene with many alleles. In the current assay, subjects must be heterozygous for A2 or A3, i.e., A2Ax or A3Ax, where Ax = other HLA-A allele. The assay measures mutant T-lymphocytes that lack a normal A2 or A3 protein due to mutation at the HLA-A gene. To identify mutants, T-lymphocytes are reacted with anti-HLA-A-A2 or -A3 antibody. Normal cells bind antibody and are killed by the addition of complement (C'), which targets antibody-bound cells. Mutant cells do not bind antibody and survive. The assay detects gene-loss/inactivating mutants and gene-duplicating mutants, shown at the lower left.

Practical Considerations in Using These Assays in Epidemiological Studies

Two biologic characteristics of cells used in any assay, their lifespan and location in the body, affect the sensitivity of the assay to the mutagenic effects of a specific exposure. The HLA-A and HPRT assays use white blood cells, specifically T-lymphocytes, which have a lifetime of several years (usually estimated as 1–4+ years) and so can accumulate HLA-A mutations over this time period. The GPA and Hb-S assays detect red blood cell mutants whose mutagenic events occurred in progenitor cells in the bone marrow, either in the differentiating cells or in the stem cells that give rise to all blood cells. For mutagenic events occurring in the differentiating cell pool, a red blood cell assay can detect effects of exposures occurring no more than to 4 to 5 months previously, as the lifespan of the mature erythrocyte is 120 days. Stem cells are long-lived, and stem cell mutants will persist for the lifetime of an individual, as demonstrated by the high GPA mutant frequencies remaining in atomic bomb survivors > 40 years after radiation exposure (32).

Location of the cells in the body also affects assay sensitivity. The GPA and Hb assays measure mutations in the bone marrow compartment only, and mutagens must penetrate to this compartment to be detected. The HLA-A and HPRT assays record mutants in T-cells in the circulating blood, so these assays may be sensitive to a wider variety of mutagens.

Practical matters of assay protocol may limit sensitivity. White blood cell (T-lymphocyte) assays require 10 to 20 mL of blood,

and the cells under test must be isolated from freshly drawn, sterile blood. These assays require cell growth, and 10 to 20 days elapse before a mutant frequency can be obtained. In contrast, the GPA assay is rapid (2 days) and requires < 1 mL of blood, which does not have to be sterile and can be analyzed for up to 2 weeks if refrigerated. In the Hb-S assay, slides can be stored at -20°C for at least 5 months (28). The GPA and HLA-A assays can be applied only to persons of certain blood types, whereas the HPRT and Hb-S assays can be used on all subjects, which may be an issue if the size of a study populations is limited.

The HPRT assay has been the most widely applied to date, and information has been developed on age as a risk factor in a sample population, on radiation and chemotherapy-exposed patients, on smokers versus nonsmokers, and on persons with cancer-prone syndromes (17-19,33-56). The GPA assay has been applied to radiation and chemotherapy-exposed individuals, persons with occupational exposures to styrene, persons with cancer-prone syndromes, and a limited general population (23,24,32,57-63). The HLA-A assay has been applied to the study of the effects of age and to chemotherapy-exposed individuals (25,27,64,65). The Hb-S assay has been applied to a small number of subjects with different mutagenic exposures (28) and with cancer-prone syndromes.

Further studies are required to supply epidemiologists with more information on the following subjects. *a)* Major risk factors for these assays, which can be confounders in a study, need to be determined. Age has been identified as a risk factor in the GPA and HLA-A assays, and both age and smoking are risk factors in the HPRT assay. *b)* The range of values for these assays among individuals in the general population (interindividual variability) needs to be characterized, as does the variation in assay values for given individuals over time (intra-individual variation). Some of this information is available for the HPRT assay (40). Subpopulations with extremely high or low assay values could help to identify important risk factors. *c)* The relation between the rate and types of somatic mutation measured in these and future assays needs to be determined relative to prior mutagenic exposures and relative to prospective risk of clinical disease. Issues such as the relation between mutations in the tissue measured and mutations in target tissues and the validity of using noncancer genes as surrogates for cancer genes need to be examined in such studies.

If further information can be collected, current and future assays of somatic mutation in humans show promise for a range of epidemiological studies: investigating cancer clusters; identifying occupational cohorts at risk; providing dose-response data for risk assessment; monitoring potentially harmful effects of clinical treatments (e.g., chemotherapy); and, through the use of intervention studies, identifying exposures that are mutagenic, and possibly carcinogenic, to humans.

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